Journal of Microbiology, Immunology and Infection (2015) xx, 1–5

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ORIGINAL ARTICLE

Molecular characteristics of *Clostridium difficile* strains from patients with a first recurrence more than 8 weeks after the primary infection

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Received 31 August 2015; received in revised form 15 October 2015; accepted 28 October 2015

Available online ■ ■ ■

KEYWORDS
antimicrobial susceptibility;
Clostridium difficile infection;
recurrence;
ribotyping

Abstract *Background/Purpose:* Nearly all published studies of recurrent *Clostridium difficile* infections (CDI) report recurrent CDI within 8 weeks after the primary infection. This study explored the molecular characteristics of *C. difficile* isolates from the first recurrent CDI more than 8 weeks after the primary infection.
Methods: Consecutive hospitalized patients with a recurrent CDI more than 8 weeks after a primary infection were enrolled prospectively from January 2008 to February 2011. All *C. difficile* isolates of the primary and recurrent infections were collected and subjected to polymerase chain reaction ribotyping and antimicrobial susceptibility testing.
Results: There were 62 cases of CDI in this study, which included 32 cases (51.6%) of recurrence due to the same ribotype of *C. difficile*, 26 (41.9%) cases due to a different ribotype, and four (6.5%) cases with 2–4 recurrences due to the same or different strains. One hundred and forty *C. difficile* isolates were obtained, which included 62 primary CDI isolates and 78 recurrent isolates. Ribotype 020 was the most common *C. difficile* strain in primary and recurrent infections. Ribotype 001 accounted for 15.4% (10/78) of recurrent infections and 3.2% (2/62) of primary infections ($p = 0.0447$). The minimum inhibitory concentration at 90% (MIC_{90})

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http://dx.doi.org/10.1016/j.jmii.2015.10.017

Please cite this article in press as: Chen Y, et al., Molecular characteristics of *Clostridium difficile* strains from patients with a first recurrence more than 8 weeks after the primary infection, Journal of Microbiology, Immunology and Infection (2015), http://dx.doi.org/10.1016/j.jmii.2015.10.017

values of linezolid, moxifloxacin, and clindamycin against type 001 strains were much higher, compared to the three other common ribotypes.
Conclusion: Recurrent CDI more than 8 weeks after a primary infection can be caused by the same or different *C. difficile* ribotype at similar percentages. Ribotype 001 *C. difficile* strains, which have a lower susceptibility to antimicrobials, were isolated more frequently in patients with a recurrent CDI.

Introduction

Clostridium difficile is a strictly anaerobic, spore-forming, Gram-positive bacillus. It colonizes the human intestinal tract and multiplies after the resident flora has been altered by treatment with broad-spectrum antibiotics. *Clostridium difficile* releases two exotoxins (i.e., A and B) and/or a binary toxin, and cause *C. difficile* infection (CDI) symptoms such as antibiotic-associated diarrhea and pseudomembranous colitis.[1,2] The epidemiology of CDI has undergone many changes since the beginning of this century. In the United States, the reported CDI incidence in 2005 (85 per 100,000 population) was nearly three times greater than the incidence in 1996 (31 per 100,000 population).[3] In the United Kingdom, CDI was listed as the primary cause of death for 3393 patients in 2006, which was seven times greater than the 499 deaths in 1999.[4] Furthermore, the endemic *C. difficile* ribotype 027 strain caused sporadic outbreaks.[4]

Clostridium difficile is highly susceptible to antibiotics that are in common use; however, treatment failure and the recurrence of infection are problematic. Even when the primary CDI is cured, recurrent CDI occurs within 8 weeks in 6–25% of patients.[5–7] For patients with at least one recurrence, the risk of a second recurrence is 45% and the risk of a third recurrence is 65%.[6,8] Recurrent CDI is a burden for patients because it increases morbidity and diminishes the quality of life in affected individuals. Furthermore, it can be fatal and has a huge economical cost.[6] The cost of recurrent CDI in the United States was US$7.1 billion in 2009, which was nearly seven times the cost for primary CDI (US $1.1 billion).[9]

Most recurrences occurred within 30 days or as late as 8 weeks after the primary infection.[6,10–14] To date, only one study has reported that CDI recurrence can occur after 8 weeks, raising the caveat that misclassification could influence epidemiological statistics.[15] We aimed to reveal the molecular characteristics of *C. difficile* isolates from patients with the first recurrent CDI more than 8 weeks after the primary CDI.

Methods

Case collection and bacterial isolates

This prospective study was conducted at the Karolinska Institutet Hospital Huddinge in Stockholm, Sweden. Consecutive cases of CDI from hospitalized patients were followed up proactively for at least 30 months. The patients with recurrence after 8 weeks were collected from January 2008 to February 2011, whereas patients without a recurrence or with a recurrence within 8 weeks of the primary CDI were excluded. All toxigenic *C. difficile* isolates (i.e., toxin B-positive, evaluated by cell cytotoxicity neutralization assay) of the primary and recurrent CDI were collected in the clinical microbiology department and stored at −80°C.

Ribotyping of C. difficile *isolates*

Bacterial cells were obtained from *C. difficile* cultured anaerobically in a peptone/yeast extract medium for 1 day. Chelex 100 resin (Bio-Rad, Sundbyberg, Sweden) was added [5% (w/v) final concentration] after centrifugation at 13,000 rpm (17,900 rcf×*g*) for 5 minutes. The mixture was heated at 96°C for 10 minutes and the template nucleic acid was obtained by another centrifugation at 13,000 rpm (i.e., 17,900 rcf×*g*) for 2 minutes. Polymerase chain reaction was targeted to the 16S-23S rRNA gene intergenic spacer region.[16] Electrophoresis and staining procedures were performed using the Genephor Electrophoresis Unit and Silver Staining Kit (GE Healthcare AB, Danderyd, Sweden). Bionumerics Software (Applied Maths, Sint-Martens-Latem, Belgium) was used to compare the molecular fingerprints with those in the local database to determine the *C. difficile* ribotypes.

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of 10 antimicrobial agents against 140 *C. difficile* isolates were determined by the agar dilution method, which followed the procedures recommended by *Approved Standard M11-A8* of the Clinical Laboratory Standard Institute (CLSI).[17] The breakpoints of metronidazole, vancomycin, rifampicin, tigecycline, and fusidic acid were referenced to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) *Clinical Breakpoint, Version 5.0*.[18] The breakpoints of moxifloxacin, clindamycin, and tetracycline were referred to CLSI M11-A8. *C. difficile* ATCC700057, *Bacteroides fragilis* ATCC25285, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC29213, and *Bacteroides thetaiotaomicron* ATCC29741 were used as reference strains.

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Statistical analysis

Descriptive analysis was used for continuous variables. Pearson's χ^2 test and Fisher's exact test were used for comparative analysis of the categorical variables. All data analyses were conducted using SPSS 18.0 software (SSPS Inc., Chicago, IL, USA) and statistical significance was set at $p \leq 0.05$.

Results

Sixty-two patients with recurrent CDI were enrolled: 51 patients had a single recurrence; eight patients, two recurrences; two patients, three recurrences; and one patient, four recurrences. The patients' ages ranged from 20 years to 99 years and 72.6% (45/62) were older than 65 years. Among the 62 patients, 32 (51.6%) patients had a total of 42 episodes of recurrent infections due to the same ribotype as the pathogen that caused the primary infection, and 26 (41.9%) patients (representing 36 episodes) had recurrent infections due to a ribotype other than that of the primary infection. Four (6.5%) patients had more than one recurrence (resenting 11 episodes in all) caused by the same or different *C. difficile* ribotype. Patients with a primary infection caused by *C. difficile* ribotype 020, SE21, or 023 more often experienced a recurrent infection due to the same type as the primary infection rather than by another ribotype. For example, eight of 10 patients infected by ribotype 020 had recurrences due to ribotype 020 and two of 10 recurrences were caused by other ribotypes. However, this difference was not statistically significant ($p = 0.097$; Table 1).

In all, 140 isolates of *C. difficile* were obtained. These included 78 recurrent isolates and 62 isolates that caused the primary infection. The latter isolates belonged to 30 ribotypes, and type 020 was most common (16.1%, 10/62), and types SE21 and 231 each accounted for 8.1% (5/62). The 78 recurrent isolates belonged to 31 ribotypes, and type 020 was most common (17.9%, 14/78), followed by ribotype 001 (12.8%, 10/78) and ribotype 023 (6.4%, 5/78). Ribotype 001 isolates were obtained from 15.4% (10/78) of recurrent isolates and 3.2% (2/62) of 62 isolates that caused primary infections ($p = 0.0447$). The highly virulent, epidemic ribotype 027 was not detected in this study.

The *in vitro* antimicrobial susceptibility study showed that all 140 *C. difficile* isolates were susceptible to metronidazole, vancomycin, and fusidic acid (Table 2). The resistance rates of rifampicin, tigecycline, tetracycline, moxifloxacin, and clindamycin was 1.4%, 1.4%, 3.6%, 14.3%, and 92.1%, respectively. The minimum inhibitory concentration at 90% (MIC_{90}) value of linezolid and fidaxomicin was 2 mg/L and 0.125 mg/L, respectively. There was no difference between the resistance rates of *C. difficile* isolates in the primary infection isolates and recurrent isolates (Table 2).

The MIC values of major ribotypes 020, 001, SE21, and 023 are listed in Table 3. The MIC_{90} values of ribotype 001 for linezolid, moxifloxacin, and clindamycin were higher than those of ribotypes 020, SE21, and 023. By contrast, the MIC_{90} value of ribotype 001 was lower than that of the other three ribotypes for fidaxomicin.

Discussion

Clostridium difficile infection is a major public health problem worldwide. Several factors are associated with CDI such as ingested *C. difficile* spores remaining in the gut, destruction of the normal flora, and a lack of specific immunity to the bacteria. However, the bacteria have lower resistance rates to commonly used antibiotics, but recurrence occurred frequently. There was no apparent correlation between recurrence and bacterial resistance.[4,12]

Almost all published studies have focused on CDI recurrence within 8 weeks after a primary infection.[6,10–14] In 1998, Wilcox et al[19] reported recurrent CDI cases in England in which the first recurrence occurred between 5 days and 2 months after a primary infection. Among 55 eligible cases, the rate of reinfection (i.e., recurrence with different ribotypes of *C. difficile*; 56%, 15/27) was numerically greater than the rate of relapse (i.e., recurrence with

Table 1 The distribution of ribotypes of 140 *Clostridium difficile* isolates causing primary infections and recurrences in 62 patients.

Primary infection		Recurrences with the same ribotype		Recurrences with different ribotype	
Ribotype	Cases *n* (%)	Cases *n* (%)	Episodes *n* (%)	Cases *n* (%)	Episodes *n* (%)
020	10 (16.1)	8 (22.2)	10 (23.8)	2 (6.7)	3 (8.3)
SE21	5 (8.1)	4 (11.1)	4 (9.5)	1 (3.3)	2 (5.6)
231	5 (8.1)	3 (8.3)	3 (7.1)	2 (6.7)	2 (5.6)
002	3 (4.8)	2 (5.6)	2 (4.8)	1 (3.3)	1 (2.8)
023	3 (4.8)	3 (8.3)	5 (11.9)	0 (0.0)	0 (0.0)
SE14	2 (3.2)	2 (5.6)	2 (4.8)	0 (0.0)	0 (0.0)
001	2 (3.2)	2 (5.6)	2 (4.8)	1 (3.3)	2 (5.6)
SE19 m	2 (3.2)	2 (5.6)	2 (4.8)	0 (0.0)	0 (0.0)
014/077	2 (3.2)	0 (0.0)	0 (0.0)	2 (6.7)	2 (5.6)
Others	28 (45.2)	10 (27.8)	12 (28.6)	21 (70.0)	24 (66.7)
Total	62 (100)	36 (100.0)	42 (100.0)	30 (100.0)	36 (100.0)

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Table 2 Comparison of antimicrobial susceptibility of 10 antimicrobial agents against 62 *Clostridium difficile* isolates causing primary infections and 78 isolates causing recurrent infections.

Antimicrobial agents	Resistance breakpoint (μg/mL)	MIC_{90} (μg/mL)			Resistance rate (%)		
		All ($n = 140$)	Primary ($n = 62$)	Recurrent ($n = 78$)	All ($n = 140$)	Primary ($n = 62$)	Recurrent ($n = 78$)
Metronidazole	>2 [a]	0.5	0.5	0.5	0	0	0
Vancomycin	>2 [a]	0.5	0.5	0.5	0	0	0
Rifampicin	>0.004 [a]	0.004	0.004	0.004	1.4	1.6	1.3
Linezolid	NA	2	2	2	—	—	—
Fidaxomicin	NA	0.125	0.125	0.125	—	—	—
Moxifloxacin	≥8 [b]	32	32	32	14.3	16.1	12.8
Clindamycin	≥8 [b]	>128	>128	>128	92.1	87.1	96.2
Tigecycline	>0.25 [a]	0.125	0.125	0.125	1.4	1.6	1.3
Tetracycline	≥16 [b]	0.25	0.5	0.25	3.6	4.8	2.6
Fusidic acid	>2 [a]	1	1	1	0	0	0

[a] Information from *Breakpoint tables for interpretation of MICs and zone diameters, version 5.0* by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[18]
[b] Information from *Methods for antimicrobialsusceptibility testing of anaerobic bacteria. Approved standard M11-A8* by the Clinical and Laboratory Standards Institute (CLSI).[17]
MIC_{90} = minimum inhibitory concentration at 90%.

Table 3 The MIC_{90} values and resistance rates of four major ribotypes of *Clostridium difficile* isolates.

Antimicrobial agents	Resistance breakpoint, μg/mL	MIC_{90} (μg/mL)				Resistance rate (%)			
		Ribotype 020 ($n = 24$)	Ribotype 001 ($n = 12$)	Ribotype SE21 ($n = 10$)	Ribotype 023 ($n = 10$)	Ribotype 020 ($n = 24$)	Ribotype 001 ($n = 12$)	Ribotype SE21 ($n = 10$)	Ribotype 023 ($n = 10$)
Metronidazole	>2 [a]	0.5	1	0.5	0.5	0	0	0	0
Vancomycin	>2 [a]	0.5	1	0.5	0.5	0	0	0	0
Rifampicin	>0.004 [a]	0.004	0.004	0.004	0.004	4.2	0	0	0
Linezolid	NA	2	8	2	2	—	—	—	—
Fidaxomicin	NA	0.125	0.032	0.125	0.125	—	—	—	—
Moxifloxacin	≥8 [b]	2	32	2	2	8.3	25	0	0
Clindamycin	≥8 [b]	16	>128	16	16	100	83.3	100	80
Tigecycline	>0.25 [a]	0.064	0.064	0.125	0.064	0	0	0	0
Tetracycline	≥16 [b]	0.125	0.25	0.125	0.125	0	0	0	0
Fusidic acid	>2 [a]	0.5	0.5	0.5	0.5	0	0	0	0

[a] Information from *Breakpoint tables for interpretation of MICs and zone diameters, version 5.0* by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[18]
[b] Information from *Methods for antimicrobialsusceptibility testing of anaerobic bacteria. Approved standard M11-A8* by the Clinical and Laboratory Standards Institute (CLSI).[17]
MIC_{90} = minimum inhibitory concentration at 90%.

the same ribotypes).[19] In 2003, Tang-Feldman et al[20] reported that relapse with the original strain occurred in 66.7% patients in the United States in which recurrence occurred mostly < 5 weeks after a primary infection.

To date, there has been a paucity of literature concerning CDI recurrence more than 8 weeks after a primary infection.[15,20] Kamboj et al[15] reported recurrence more than 8 weeks; however, specific *C. difficile* ribotypes were not identified. In this study, a similar percentage of recurrent CDI occurring more than 8 weeks after a primary infection was caused by the same ribotype isolate (51.6%) or a different ribotype isolate (41.9%). The most common *C. difficile* ribotype was 020 in the primary and recurrent CDIs. The patients whose primary infections were caused by ribotype 020 were more likely to have a recurrence with the same ribotype. Magnusson et al[21] reported that ribotype 001 was the most common isolate in nosocomially acquired CDIs, and it was prone to cause relapse (i.e., recurrence with the same ribotypes). Ribotype 001, the second most common ribotype in recurrent infections in this study, was isolated more frequently in recurrent infections (15.4%) than in primary infections (3.2%, $p < 0.05$).

The drawbacks of this study are that the clinical data of patients with a recurrent CDI more than 8 weeks after the

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primary infection were unavailable; therefore, the clinical characteristics and prognosis of these patients in comparison to patients with recurrent CDI within 8 weeks were lacking.

All 140 *C. difficile* isolates in this study were susceptible to metronidazole and vancomycin, which is consistent with the finding in other reports.[4,22] The MIC_{90} values of ribotype 001 to linezolid, moxifloxacin, and clindamycin were higher than those of the other ribotypes. A recent study demonstrated that low-molecular-weight S layer proteins of ribotype 001 strains showed 88% identity with the proteins of ribotype 027, which is a hypervirulent strain, and causes outbreaks and recurrent CDIs worldwide.[23] Furthermore, low-molecular-weight S layer proteins are immunodominant antigens, which may have a role in attacking hosts.[23]

In summary, a recurrent CDI more than 8 weeks after the primary infection could be caused by the same or different *C. difficile* ribotypes at similar percentages. Ribotype 001 *C. difficile* strains were isolated more frequently in recurrent CDI patients. Further studies are needed to explore the clinical characteristics of patients with recurrent CDI occurring more than 8 weeks after the primary infection, compared to the characteristics of patients with recurrent infection occurring within 8 weeks.

Conflicts of interest

All contributing authors have no conflicts of interest to declare.

Acknowledgements

This work was supported by the Scandinavian Society for Antimicrobial Chemotherapy and a grant from National Natural Science Foundation of China (Grant No. 30973594).

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